Use of an Image Analysis System to Karyotype Diploid Alfalfa (*Medicago sativa* L.)

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Alfalfa somatic chromosomes are difficult to karyotype due to autotetraploidy, a relatively large number of chromosomes ($2n = 4x = 32$), small chromosome size (2 to 3 μm), and difficulty in identifying all eight sets of chromosomes because of their similar chromosome morphology. These difficulties can be surmounted by studying diploid ($2n = 2x = 16$) alfalfa and using a microcomputer-based image analysis system for karyotyping. Image analysis enhances the chromosome image by pseudocolorizing the image according to densitometric measurements and by enlarging the image, which enables the edges of the chromosomes to be distinguished. The chromosome arms from 26 cells were measured, and the relative chromosome lengths and arm ratios were computed. A randomized complete block design experiment showed that the relative length and total chromosome length measurement could be used to distinguish the eight homologous chromosome pairs. The video image was cut and pasted on the video screen according to the analyzed data to produce the karyotype. The diploid alfalfa karyotype consists of one pair of satellited chromosomes, one pair of large submetacentric chromosomes, three pairs of metasubmetacentric chromosomes, and three pairs of small metacentric chromosomes. This image analysis system is especially useful for plant species such as alfalfa that have been difficult to karyotype due to small chromosome size, similar chromosome morphology, and polyploidy. Other applications of this image analysis system are discussed.

Alfalfa (*Medicago sativa* L.) somatic chromosomes are difficult to karyotype due to their autotetraploidy, the relatively large number of chromosomes ($2n = 4x = 32$), the small chromosome size (2 to 3 μm), and the difficulty in identifying all eight sets of chromosomes due to their similar morphology (McCoy and Bingham 1988). Various researchers (Agarwal and Gupta 1983; Buss and Cleveland 1968; Falistocco 1987; Schlarbaum et al. 1988) have published karyotypes of diploid and tetraploid alfalfa; however, multiple chromosome spreads were not analyzed, and either direct measurements were taken using an ocular micrometer or photomicrographs were measured. The difficulties in karyotyping autotetraploid alfalfa can be overcome by studying diploid ($2n = 2x = 16$) alfalfa: this species has half the number of chromosomes; there are only two genomes; and, by doubling the chromosome number, an autotetraploid can be produced. Image analysis methods may be used to overcome the difficulties of visualizing small chromosomes, and precise measurements of the chromosomes can be obtained for karyotypic analysis.

Image analysis of biological specimens utilizing video images has been used to enhance images for more critical observations and to collect morphometric data since the early 1970s (reviewed by Inoue 1988). However, until recently cytogeneticists have not taken advantage of this technology to collect morphometric data and to increase the efficiency in cytological, microscopic, and photographic procedures to karyotype chromosome genomes. The first image analysis system for chromosomes was developed for the karyotypic analysis of humans (Castleman and Melnyk 1976). Since that time, several commercial systems have been released for human karyotypic analysis (Finnon et al. 1986; Taylor and Graham 1980). Fukui (1985) was the first to develop an image analysis system to karyotype plant chromosomes. These systems have required the use of either large mainframe computers or minicomputers that are typically too expensive for the average cytogenetic laboratory budget.

The rapid development of microelectronics and computer programming has recently made it possible at a much re-

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Journal of Heredity 1994;85:18-22; 0022-1503/94/$5.00

duced cost for personal microcomputers to accomplish the task that formerly could only be done by larger computers. Such a system based on a high-resolution video camera and a microcomputer has been developed by Loats Associates, Inc. (Westminster, Maryland).

The purpose of this study was to (1) develop a software program in cooperation with Loats Associates for constructing plant chromosome karyotypes, (2) determine the efficacy of the image analysis system to differentiate between alfalfa chromosomes, and (3) develop a karyotype of diploid ($2n = 2x = 16$) alfalfa.

Materials and Methods

Germplasm Source and Cytological Techniques

We obtained seed of diploid $2x$ iso CADL (cultivated alfalfa at the diploid level) from Ted Bingham (University of Wisconsin, Madison). Roots from germinating seeds were placed in ice water for 22 h, fixed in Farmers Fixative (3:1 mixture of 95% ethanol : glacial acetic acid), and stored in the refrigerator for at least 24 h. Root tips were hydrolyzed for 10 min in 1N HCl at 60°C, placed in Feulgen's stain for at least 3 h, and squashed in 1% acetocarmine. Observations were made using a Zeiss Universal microscope with the image analysis video camera attached to the microscope. We analyzed 26 well-spread diploid cells.

Image Analysis System Description

The image analysis system is based on an AT&T PC with an 80386 microprocessor running at 20 MHz clock speed with 32-bit architecture. The computer has an Intel 80387 math coprocessor, 512 KB base RAM with 4 MB extended RAM memory, 135 MB hard drive, 1.44 MB and 1.2 MB floppy drives, an IOmega dual cartridge 20 MB Bernoulli Box, Sony Trinitron multi-input CGA text and NTSC video display monitor, Mitsubishi RGB analog color monitor, GTCO Corporation four-button Digi PAD mouse, Polaroid Freeze Frame video photographic system, and an Epson FX-286 dot matrix printer.

The video digitizing components consist of a specially configured black and white Dage 68 video camera. Image acquisition is enabled by a high-resolution frame grabber (digitizing board) with eight 256 by 8-bit RAM input look-up tables, two 512 by 512 by 8-bit high-speed frame-store memory buffers, eight 256 by 24-bit RAM output look-up tables, and three 8-bit video RGB outputs (one each for red, green, and blue). An entire video frame is digitized in 1/30 of a second. Digitized means that each pixel (one of 245,760 spots which compose the video picture) is assigned a value of 1–256, depending on its densitometric measurement. Thus, the image is converted into a matrix of densitometric measurements. The NTSC video signal is converted at 10 MHz into 480 lines by 512 pixels per line by 8 bits per pixel. The 8-bit information for each pixel represents one of 256 possible gray levels which can be assigned various colors to present a pseudocolorized image. The program uses a ratio illumination correction procedure based on the digital image of the background without the target image. The image display subsystem is based on a 512 by 480 by 8 bit architecture (245,760 pixels with 256 colors) and uses a 1 MB RAM display buffer. The standard output is analog-RGB and is displayed on a high-resolution analog color monitor. The monitor is divided into four quadrants that can display up to four images at one time.

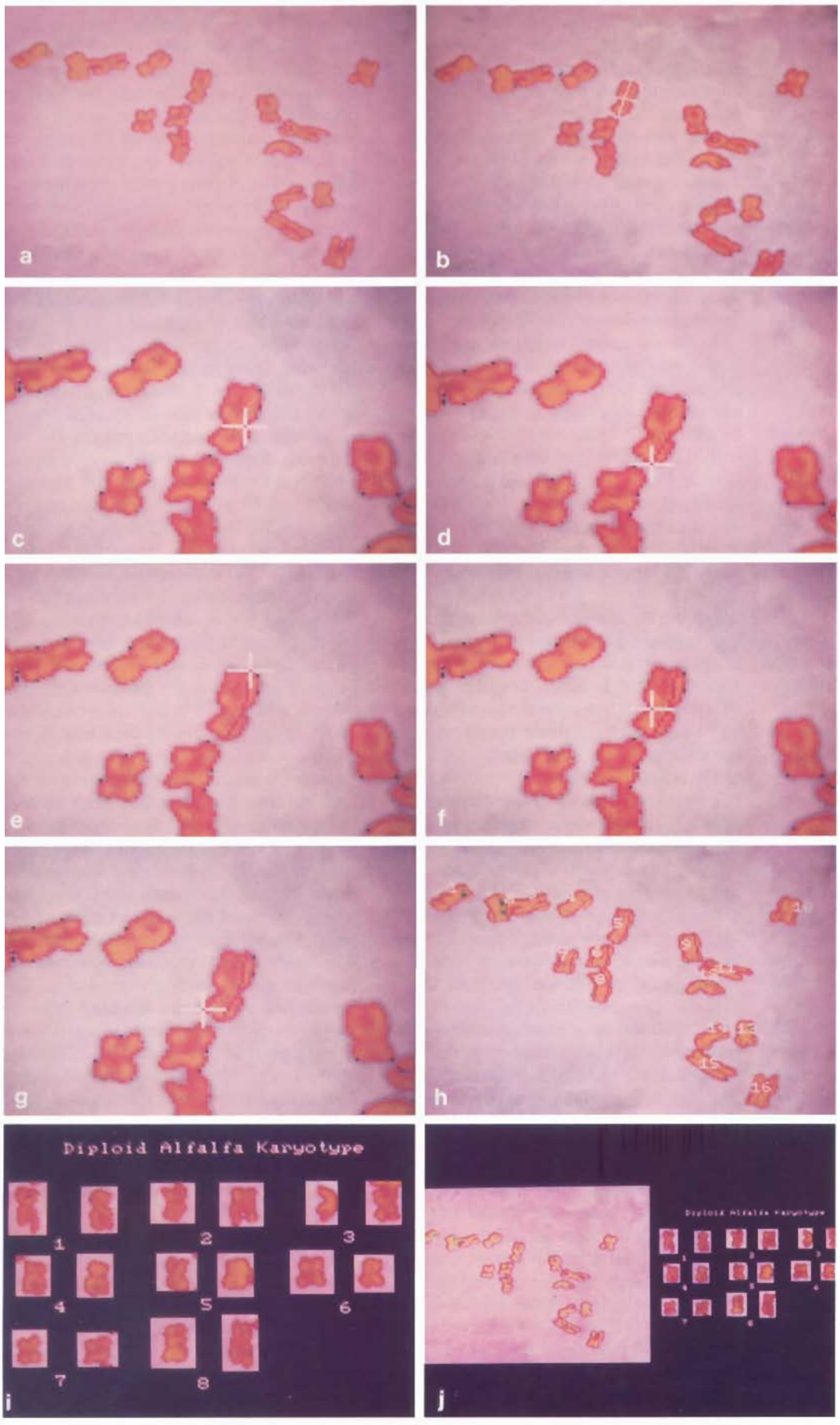
Software for Karyotypic Analysis

The software was written in C language by Loats Associates, in cooperation with Gary Bauchan. The software program is only compatible with the custom-designed video camera and digitizing board also developed by Loats Associates. Karyotypic analysis was divided into five main steps: (1) digitization and storage of video images, (2) morphometric measurements of chromosome morphology, (3) analysis of data, (4) construction of the karyotype, and (5) output of results.

Digitization and storage of images. We identified the chromosome spreads to be karyotyped by observing the chromosome squashes on the microscope slides, using first the 16× objective then the 63× or 100× oil-immersion objectives on the microscope. Once a well-spread cell was identified, the image was placed out of focus and digitized. This image was stored and used as the background image. The background image was automatically subtracted from the chromosome image to eliminate background noise. We then placed the chromosome spread in focus, and the video image was digitized using the frame grabber video board. The digitized image was initially stored on the hard disk. Each digitized image required 66 K memory; thus images were archived either onto a 20 MB Bernoulli cartridge or floppy disk.

Morphometric measurements of chromosome morphology. We recalled stored images of a stage micrometer using each of the available microscope objectives; the instrument was calibrated by determining the number of pixels between a micrometer unit, and the actual value of each pixel was determined by division. The digitized chromosome spreads were recalled and displayed in the first quadrant on the RGB analog color monitor. Initially, we performed a gray-level compression (that is, gray levels above and below a particular level were not displayed) on the image to pseudocolor-enhance the contours of the chromosomes (Figure 1a). The program text screen on the monitor prompts the operator, "How many chromosomes are in the spread?" which allows the program to set up the second monitor quadrant to receive rotated chromosome images. The cell was then displayed full screen, which represents a 4× magnification. The digitized image can be enlarged to a maximum of 16× by using the zoom feature to enhance chromosome images. Interactively using the GTCO Digi PAD mouse and the keyboard while viewing the digitized image, we rotated the pointer in the shape of a cross (Figure 1b) such that the short arm of the chromosome was rotated to the top of the image. When the rotation was completed, a copy of the individual chromosome image was stored in a box in the second quadrant (Figure 1i). Using the mouse, we located the centromere on the first chromatid (Figure 1c), then located the short arm (Figure 1d) and the long arm (Figure 1e). We repeated this for the second chromatid, identifying the centromere (Figure 1f) and each arm (Figure 1g). If the chromosome was not straight, we selected several points and determined the total length along this curve, repeating this procedure for each chromosome. The number of pixels were counted between the points selected; the calculated value of each pixel as determined by calibrating the instrument initially was computed; and the measurement was displayed on the program's text screen. The program allows the operator to number the chromosomes on the screen in the order in which the chromosome measurements were taken (Figure 1h).

Analysis of the data. The data screen displayed the chromosome's number, length of each short arm, length of each long arm, length of the satellite (SAT) if applicable, the average measurement of the short arms per chromosome, the average measurement of the long arms per chromosome, the average length of the SAT if applicable, the arm ratio (long arm average/short arm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average), total length of the chromosome (short arm plus long arm), and relative chromosome length (length of the individual chromosome/total of all chromosome lengths). We rearranged the data table by sorting the table according to the total chromosome lengths in descending order from longest to shortest chromosome to aid in identifying homologous pairs.

Construction of the karyotype. We used the rotated chromosomes appearing in the second quadrant to develop the karyotype. Using the analyzed data to identify homologous pairs, we rearranged the chromosomes using the mouse to interactively cut and paste the chromosome images. We arranged the chromosomes from longest to shortest (Figure 1i–j). We typed labels on the keyboard and used the mouse to place them into the image (Figure 1i–j). Images of the karyotype were stored on the hard disk for later recall.

Output of results. Using the dot matrix printer, we printed the data from the morphometric measurements or we stored them on the hard drive for later analysis. Photographs were taken of the digitized RGB analog image using a Polaroid Freeze Frame video camera system. Resolution of the freeze frame is 650 by 500 lines. An instant 4 in × 3 in print using Polaroid Type 339 color film was used as a quick record of the chromosome image with the chromosomes numbered in the order in which the data were collected. Kodak Ektachrome ASA 100, 35 mm slide film was used to make a permanent record of the original cell and the karyotype. Cibachrome prints were made directly from the slides. The entire image analysis system, hardware and software, can be purchased from Loats Associates at an approximate cost of $60,000 U.S.

Efficacy of the Image Analysis System

The efficacy of the image analysis system to differentiate between alfalfa chromosomes was tested on 26 cells from diploid 2*x* iso CADL. We analyzed individual chromosomes immediately after preparation and again 1 week later. The experimental design was a randomized complete block with 26 replications (cells) and eight treatments (chromosome sets). Cells were chosen at random, but the chromosome sets were fixed (homologous chromosomes). We obtained short arm length, long arm length, SAT length, arm ratio, total chromosome length, and relative chromosome length. A repeated measure ANOVA (SAS Institute 1989) was conducted on each response variable.

Table 1. Mean squares from a repeated measure ANOVA of the efficacy of an image analysis system for differentiating alfalfa chromosome sets

Source	df	Length			Arm ratio	Relative length
		Long arm	Short Arm	Total		
Between cell and chromosome sets effects						
Cells	25	11,707.44**	5,234.62**	33,370.11**	2,216.12	260.76
Chromosome sets	7	14,341.55**	3,156.87**	30,354.65**	7,464.65**	15,796.46**
Error	175	208.71	154.37	811.55	134.31	515.98
Within cell and chromosome sets effects						
Time	1	100.09	59.25	1,403.12	1,037.62	532.14
Time × cells	25	280.71**	112.95**	979.21**	944.49	299.19
Time × chromosome sets	7	46.98	62.70	635.45	1,411.15	325.82
Error (time)	175	45.83	46.79	502.27	878.88	378.29

*, ** Significance at the .05 and .01 probability levels, respectively.

Results and Discussion

The image analysis system with the karyotyping software is an efficient method of obtaining high-quality images of alfalfa chromosomes through the use of enhancement techniques. Enhancement of the chromosomes by pseudocoloration and enlargement of the images enables the edges of the chromosome to be distinguished for easy identification of the centromere and of the ends of each arm of the chromosome (Figure 1a–h). The image analysis system is also a rapid method of obtaining large amounts of data on chromosome arm lengths, arm ratios, and relative chromosome lengths from several cells. The average time spent measuring a cell from the time a cell is identified as a good spread is 20 min. The only limitation is the initial preparation of well-spread chromosome squashes. The repeated measure ANOVA indicated that it did not matter when the measurements were taken—either immediately or 1 week later—the pairing of the homologous pairs remained the same (Table 1). The efficacy of the image analysis system for differentiating among chromosome sets showed that the total length of the chromosome and relative chromosome length can be used to distinguish homologous pairs. Coefficients of variation indicate that parameter estimation was reasonably precise (Table 2). The cell selected to exhibit the karyotype of diploid alfalfa using the image analysis system was judged to be the best representative of the 26 cells analyzed. The diploid alfalfa karyotype consists of one pair of satellited (SAT) chromosomes (chromosome pair #8), one pair of large submetacentric (sm) chromosomes (chromosome pair #1), three pairs of metasubmetacentric (msm) chromosomes (chromosomes 2–4), and three pairs of small metacentric (m) chromosomes (chromosomes 5–7) (Table 2). Chromosome #8 contains the nuclear organizer region (NOR) region of the alfalfa genome, and the SAT chromosomes are a benchmark feature of the karyotype. The large submetacentric chromosome pair #1 is easy to distinguish if the chromosomes have not constricted too much during pretreatment. Occasionally a tertiary constriction can be found on the metasubmetacentric chromosome set #4. This was also observed by Schlarbaum et al. (1988).

The karyotype developed from this study is comparable to the other karyotypes published (Agarwal and Gupta 1983; Buss and Cleveland 1968; Falistocco 1987; Schlarbaum et al. 1988). The cells analyzed here were all obtained from plants from a single germplasm source. Knowing that alfalfa is an outcrossing species, intraspecific variation must be recognized

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Figure 1. Karyotypic analysis of a diploid alfalfa (*M. sativa*) using image analysis: (**a**) Root tip cell with 16 chromosomes which have been pseudocolor enhanced; (**b**) Rotation of the cross/circle cursor so the short arm of the chromosome is at the top of the cursor; (**c**) Identification of the centromere after a 4× increase in magnification; (**d**) Measurement of short arm length; (**e**) Measurement of long arm length; (**f**) Identification of the centromere on the second chromatid; (**g**) Measurement of the short arm on the second chromatid; (**h**) Numbering of the chromosomes in the order in which the measurements were taken; (**i**) Cut and pasted image of the diploid alfalfa karyotype; (**j**) Image of the original cell on the left and the karyotype of that cell on the right.

Table 2. Efficacy of the image analysis system for differentiating among chromosome sets in altalfa[a]

Chromosome set	Length				Arm ratio	Relative length	Centromere position[b]
	Long Arm	Short Arm	SAT	Total			
1	1.38a	0.89a		2.27b	1.58a	15.3a	sm
2	1.21b	0.84a		2.05c	1.56a	13.9b	msm
3	1.17b	0.77b		1.94d	1.47abc	13.1c	msm
4	1.09bc	0.76bc		1.85e	1.46abc	12.5d	msm
5	1.00d	0.75bc		1.76f	1.36bcd	11.9e	m
6	0.92e	0.74bc		1.66g	1.31cd	11.2f	m
7	0.85f	0.65d		1.50h	1.26d	10.1g	m
8	1.05cd	0.71c	0.65	2.40a	1.51ab	15.4a	SAT
CV[c]	12.8	15.9		9.1	21.5	9.0	

[a] Means not followed by the same letter are significantly different at the .05% level based on Tukey's test.

[b] Centromere designation adapted from Levan et al. (1964): SAT = satellited; sm = submetacentric; msm = metasubmetric; m = metacentric.

[c] CV = coefficient of variation.

when making comparisons of karyotypes. The karyotype constructed using image analysis conforms to the standardized karyotype for diploid pachytene chromosomes of alfalfa (Kasha et al. 1970).

This study showed that image analysis techniques are a reliable and efficient method of karyotyping diploid alfalfa. In fact, previously, it has been practically impossible to obtain numerical data on the arm length, arm ratios, and especially total arm length to accurately karyotype diploid alfalfa. Difficulties still arise when attempting to karyotype a particular cell. The similarity of chromosomes 2 and 3 plus the small metacentric chromosomes (5, 6, and 7), makes karyotyping difficult. Chromosome banding techniques have been attempted, but the heterochromatinized areas of the chromosomes appear only at the centromere, and the patterns have not proven useful in distinguishing individual chromosomes (Schlarbaum et al. 1988). Tagging of individual chromosomes using in situ hybridization techniques has been successful in alfalfa (Schaff et al. 1990), but DNA probes that label the chromosome differentially are not yet available for all eight pairs of chromosomes. This image analysis system is especially useful for plant species such as alfalfa that have been difficult to karyotype due to small chromosome size, similar chromosome morphology, and polyploidy. Karyotypic analysis using image analysis can be used to (1) karyotype tetraploid cultivated alfalfa, (2) identify aneuploid chromosomes in trisomic and nullisomic cells, (3) determine genetic linkage through the use of in situ hybridization techniques, and (4) study evolutionary relationships among species of *Medicago*.

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